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Eriodictyol induces apoptosis *via* regulating phosphorylation of JNK, ERK, and FAK/AKT in pancreatic cancer cells

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Abstract Although it has been intensively studied over the past few decades, pancreatic cancer remains one of the most lethal cancers. Eriodictyol, a plant-derived flavonoid mainly found in citrus fruits, exerts diverse biological effects, including anti-oxidant, anti-cancer, and anti-inflammatory properties. In this study, we investigated the anticancer properties of eriodictyol and its mechanisms of action in pancreatic cancer cells. In both SNU213 and Panc-1 cells, eriodictyol decreased viability, induced apoptosis, and decreased clonogenicity. In addition, eriodictyol treatment increased the phosphorylation level of JNK and decreased the phosphorylation levels of ERK, FAK, and AKT. These observations provide insight into the molecular mechanisms of eriodictyol-induced apoptosis in pancreatic cancer cell lines, and could contribute to the development of candidate compounds for treating pancreatic cancer.

Keywords Apoptosis · Eriodictyol · FAK/AKT · MAPK · Pancreatic cancer

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Introduction

Cancer is the second leading cause of death in the United States. Pancreatic cancer is an especially deadly disease, with a 5-year survival rate less than 8% [1], and despite efforts to improve treatment, it is expected to become the second leading cause of cancer death by 2030 [2]. Surgery followed by adjuvant chemotherapy is still an effective treatment for localized pancreatic cancer; however, metastasis has often occurred by the time of diagnosis, so only 15-20% of patients with pancreatic cancer are eligible for surgery/chemotherapy [3]. Gemcitabine and 5-fluorouracil are primary chemotherapy drugs for patients with pancreatic cancer, but the prognosis remains poor due to drug resistance and side effects [4-5]. Consequently, drugs that are safer and more effective than currently available chemotherapeutic agents are urgently needed.

For many centuries, natural products have been used to treat a wide range of diseases, and thus represent a reservoir of new, structurally diverse compounds with interesting biological effects [6-8]. According to a report by the World Health Organization, ~65% of the world's population still depends on plant medicines for their health care [9].

Flavonoids are a major class of plant secondary metabolites found in vegetable- and fruit-derived foods such as apple, grapes, garlic, red wine, and green tea [10-11]. Flavonoids have roles antioxidant, anti-inflammatory, and antibacterial agents. Among the flavonoids, quercetin increases antioxidant by regulating glutathione and reactive oxygen species [12]. Curcumin has anti-inflammatory effects in chronic disease [13] and kaempferol has antidiabetic effects by inhibiting gluconeogenesis [14]. Flavonoids also improve drug bioavailability in cancer and play roles in cell-cycle interference, induction of apoptosis induction, and inhibition of angiogenesis [15-18]. Eriodictyol [2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-2,3-dihydrochromen-4-one; fig. 1A] is a flavanone found in fruits such as lemon (*Citrus limon* BURM. f.) [19], sutil lemon (*Citrus aurantiifolia*) [20], and bergamot orange (*Citrus bergamia* Risso) [21]. Eriodictyol has anticancer effects in liver,

lung, and colorectal cancer cells [22-24], but its effects in pancreatic cancer remain unknown. Hence, in this study, we investigated the mechanisms associated with the anticancer effect of eriodictyol in pancreatic cancer cells.

Materials and Methods

Cell culture conditions

Human embryonic kidney cells (293T) and human pancreatic cancer cells (Panc-1 and SNU-213) were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). 293T, Panc-1, and SNU-213 cells were cultured as previously described [25]. Eriodictyol (Cat. No. 020056) were purchased from Indofine Chemical Company (Hillsborough, NJ, USA).

Cell viability assay

Cell viability was measured using WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl) 2H-tetrazolium] solution (Boehringer Mannheim, Mannheim, Germany). Briefly, cells were seeded at a density of 2.5×10^4 cells/well in 24-well plates and incubated for 24 h. The cells were treated with eriodictyol in DMEM at various concentrations (5, 10, 25, 50, and 100 μM) for 72 h at 37 °C. After treatment with WST-1 solution, absorbance was measured at 450 nm on a Multiskan Spectrum microplate reader (Thermo Fisher Scientific, Vantaa, Finland).

Colony formation assay

Panc-1 and SNU-213 cells were seeded in 60-mm dishes at a density of 200 cells/dish. After 24 h incubation, the cells were treated with various concentrations of eriodictyol (0, 25, and 50 μM) for 10-14 days at 37 °C in a humidified atmosphere containing 5% CO_2. Colonies were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet solution for 15 min, and then washed with sterile deionized water. Visible colonies were counted.

Flow cytometry analysis

293T, Panc-1, and SNU-213 cells were seeded in 6-well plates and incubated for 24 h. The cells were treated with eriodictyol for 72 h, and then incubated with annexin V-FITC and propidium (PI) (FITC Annexin V apoptosis detection kit, BD Pharmingen, San Diego, CA, USA). Apoptotic cells were detected by flow cytometry on a LSRFortessa instrument (BD Pharmingen).

Western blot assay

Cells were lysed in M-PER lysis buffer (Thermo Fisher Scientific, Bonn, Germany) containing protease inhibitor cocktail (Hoffmann-La Roche Ltd, Base, Switzerland), 2 mM sodium vanadate, 30 mM sodium pyrophosphate, and 100 mM sodium fluoride. After quantification, proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK). Primary Antibodies against cleaved PARP, p-FAK (Tyr397), p-AKT (Ser473), p-JNK (Thr183/Tyr185), p-ERK1/2 (Thr202/Try204), and GAPDH were obtained from Cell signaling Technology (Danvers, MA, USA). Membranes were blocked with 5% skim milk in TBST and incubated with primary antibodies at were diluted 1:1000 in TBST and incubated with the membranes overnight at 4 °C. Secondary antibodies (Merck Millipore, Darmstadt, Germany) were diluted 1:4000 in TBST and incubated with the membranes for 1 h. Protein bands were detected using an ECL kit (Biosesang, Seongnam, Korea).

Statistical analysis

Error bars represent ± SEM. Statistical analysis was performed by one-way ANOVA and differences among multiple groups were analyzed using Turkey's post hoc method. $p < 0.05$ indicated significant differences.

Results

Eriodictyol decreases the viability of pancreatic cancer cells

To investigate the effect of eriodictyol on the viability of pancreatic cancer cells, we treated human pancreatic cancer cells (Panc-1 and SNU-213) with eriodictyol at various concentrations and measured viability by WST-1 assay (Fig. 1). Human embryonic kidney cell (293T) was used as a control. The viabilities of pancreatic cancer cells decreased in a dose-dependent manner. Treatment with 50 μM eriodictyol decreased cell viability by 33% in Panc-1 and 44% in SNU-213 relative to the corresponding untreated controls. However, eriodictyol did not have a significant effect on 293T cells, demonstrating that its cytotoxicity was selective for pancreatic cancer cells. Next, to determine the-tumor suppressive effect of eriodictyol, we performed colony formation assays. Eriodictyol significantly decreased colony formation in Panc-1 and SNU-213, suppressing clonogenicity (Fig. 2).

Eriodictyol induces apoptosis in pancreatic cancer cells

We then performed flow cytometry to investigate the effects of eriodictyol on apoptosis. Eriodictyol induced apoptosis in a dose-dependent manner: the percentage of apoptotic cells increased from 7.1% in untreated Panc-1 cells to 27.2% in cells treated with 50 μM eriodictyol. In SNU-213, eriodictyol increased the proportion of apoptotic cells even further, from 8.7 to 41.2% (Fig. 3A). By contrast, eriodictyol treatment had very little effect on 293T control cells.

The nuclear protein poly (ADP-ribose) polymerase (PARP) helps cells to maintain their viability, and the cleaved form of PARP serves as a marker for mitochondria-mediated apoptosis [26,27]. Eriodictyol treatment increased the level of cleaved PARP in a dose-dependent manner in both Panc-1 and SNU-213 cells, demonstrating that this compound can induce apoptosis in pancreatic cancer cells (Fig. 3B).

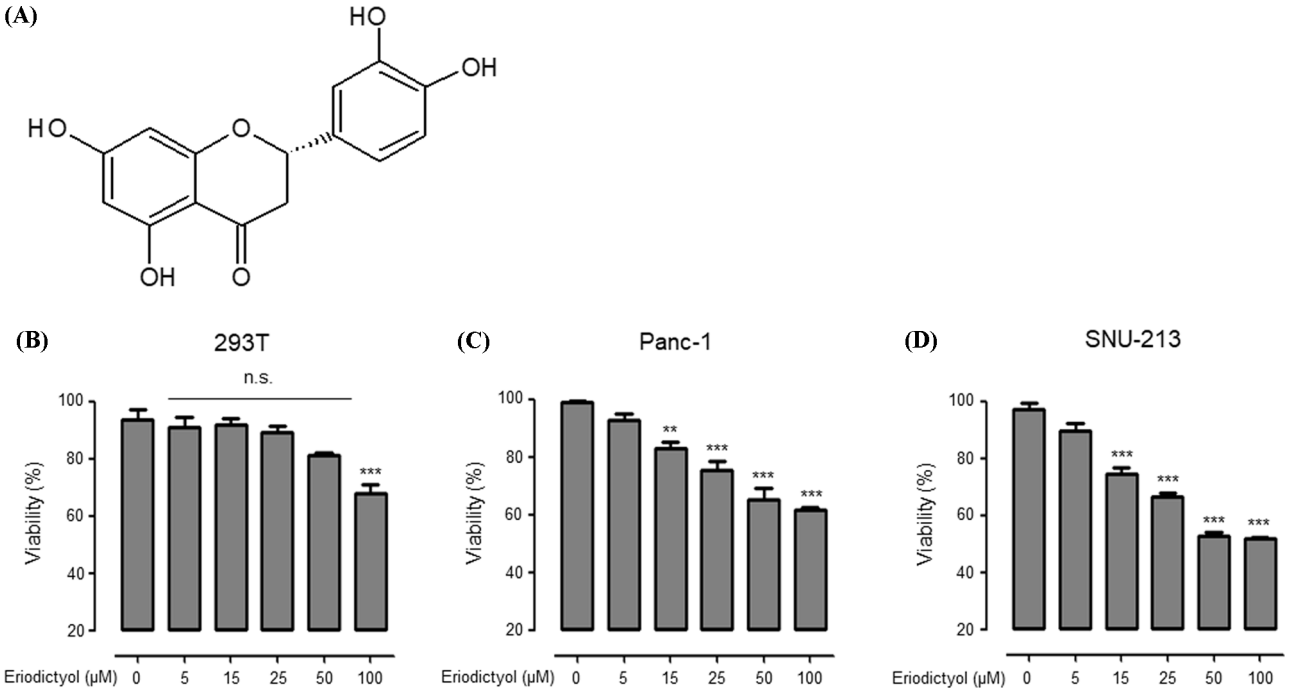

Fig. 1 Effects of eriodictyol on the viability in human pancreatic cancer cells. (A) Chemical structure of eriodictyol. (B) Human embryonic kidney cells (293T) and human pancreatic cancer cells (Panc-1 and SNU-213) were treated with various concentrations (0, 5, 15, 25, 50, and 100 μM) of eriodictyol for 72 h. Cell viability was determined by WST-1 assay. Graphs show means ± SEM, *p <0.05; **p <0.01; ***p <0.001 vs. control group

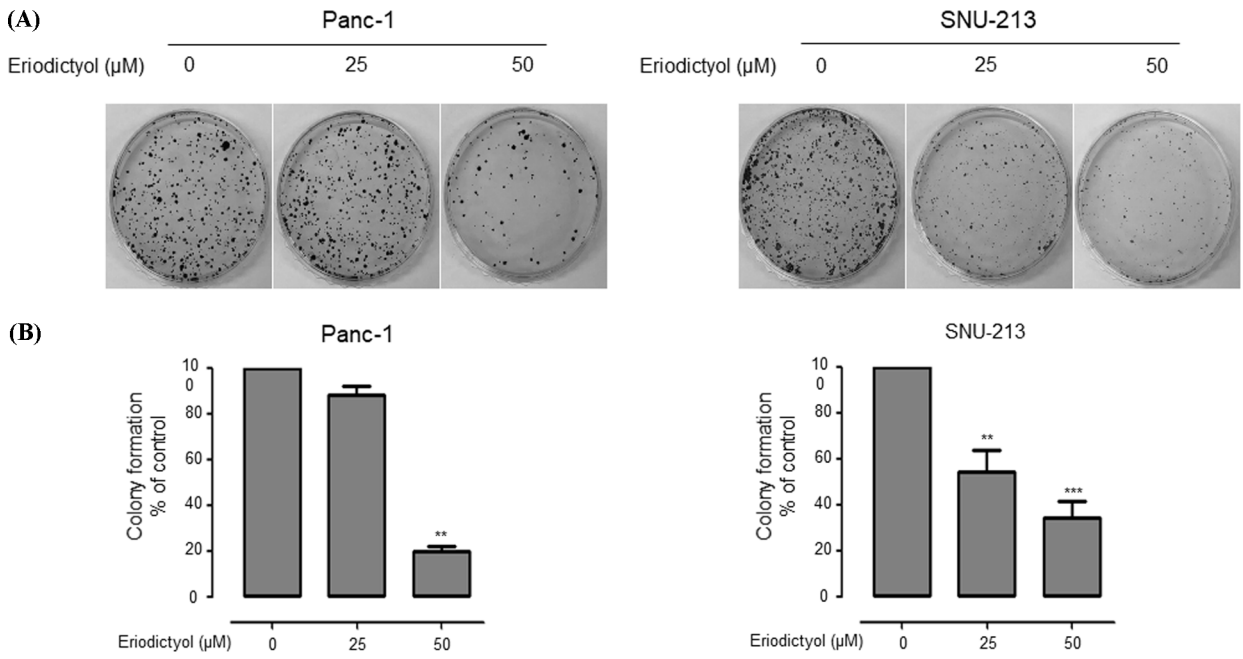

Fig. 2 Eriodictyol suppresses colony formation by human pancreatic cancer cells. (A) Colony formation assay. Panc-1 and SNU-213 cells were treated with 0, 25, or 50 μM Eriodictyol for 24 h. In both cell lines, eriodictyol significantly attenuated colony formation in a dose-dependent manner. (B) Colonies were counted and graphed. Data represent means ± SEM. *p <0.05; **p <0.01; ***p <0.001 vs. control group

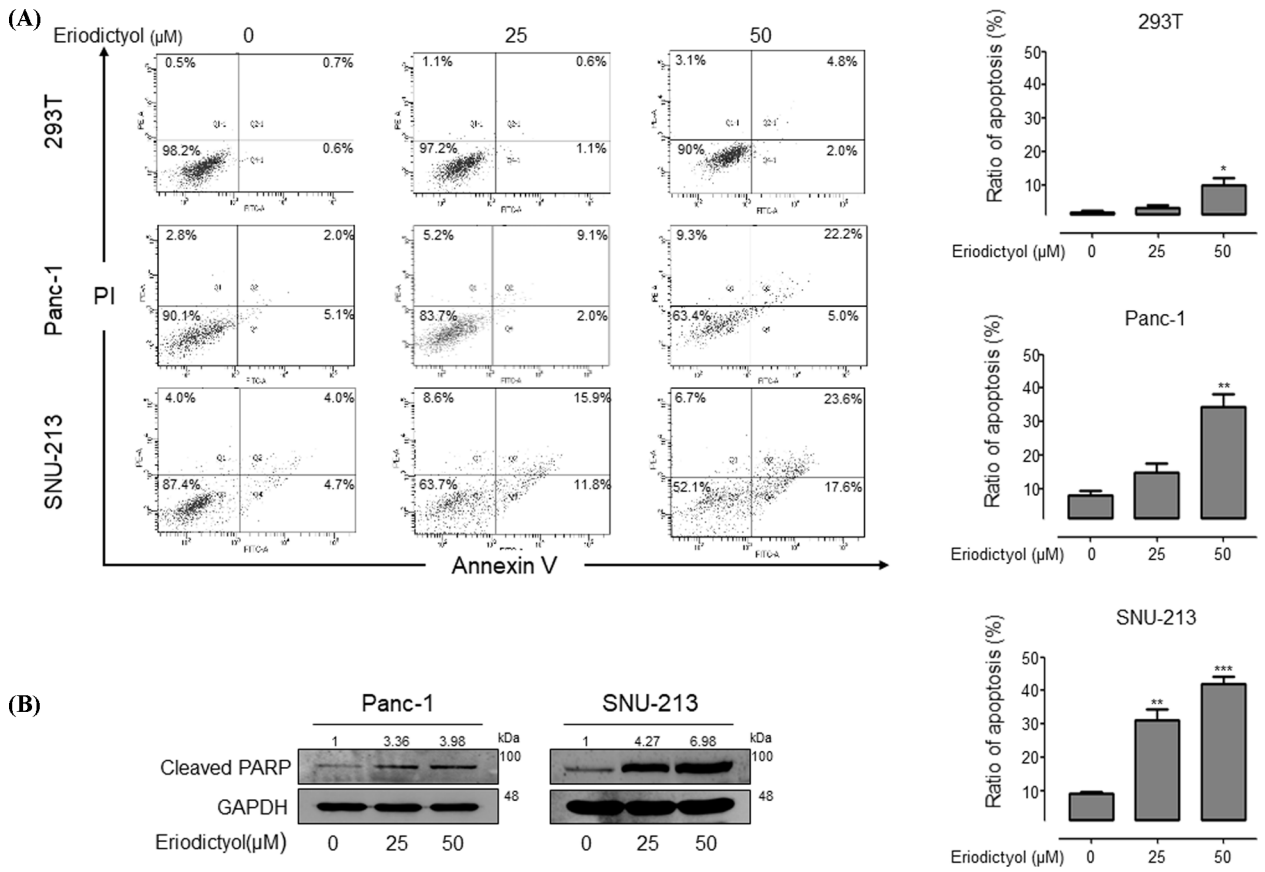

Fig. 3 Effects of eriodictyol on apoptosis in human pancreatic cancer cells. (A) Left: representative flow cytometry dot plots of apoptosis assays of eriodictyol-treated 293T, Panc-1, and SNU-213 cells. Cells were incubated with 0, 25, or 50 μM eriodictyol for 72 h. Cells were collected, stained with Annexin V-FITC and PI, and detected by flow cytometry. Right: relative percentages of apoptotic cells. Graphs show means ±SEM, *p <0.05; **p <0.01; ***p <0.001 vs. control group. (B) Western blot analysis of apoptosis-related proteins after treatment of Panc-1 and SNU-213 with 0, 25, or 50 μM eriodictyol for 48 h. Cell lysates were subjected to SDS-PAGE, following by western blotting with antibodies against cleaved PARP and GAPDH

Eriodictyol induces apoptosis of pancreatic cancer cells via the MAPK and FAK/AKT signaling pathways

To elucidate the mechanisms of action of eriodictyol in pancreatic cancer cells, we examined the phosphorylation levels of JNK and ERK in Panc-1 and SNU-213. Sustained activation of JNK induces apoptosis [28]. Eriodictyol treatment (25 μM) significantly increased the phosphorylation levels of JNK in both cell lines (Fig. 4A). In addition, the phosphorylation level of JNK increased in a time-dependent manner in both cell types after treatment with 50 μM eriodictyol (Fig. 4B). ERK signaling, which is active in pancreatic cancer, is strongly associated with cell proliferation [29]. In contrast to JNK, phosphorylation of ERK decreased following eriodictyol treatment.

Because activation of the FAK/AKT signaling pathway is related to the viability and metastasis of pancreatic cancer cells [27,30], we next examined the phosphorylation levels of FAK and AKT. Figure 4C shows that eriodictyol inhibits the phosphorylation level of FAK and AKT in Panc-1 and SNU-213 cells. Furthermore, the phosphorylation levels of FAK and AKT were decreased in a time-dependent manner in Panc-1 and SNU-213 cells after 50 μM eriodictyol treatment (Fig. 4D). Together, these data demonstrate that eriodictyol regulates the phosphorylation of JNK, ERK, and FAK/AKT to induce apoptosis in pancreatic cancer cells.

Discussion

In this study, we found that eriodictyol treatment selectively decreased pancreatic cancer cell viability by inducing apoptosis. Apoptosis is one of major processes by which harmful cells are eliminated from the body. Accordingly, apoptosis is also closely linked to the incidence and progression of cancer and the process is characterized by the activation and inactivation of certain proteins [31]. PARP plays an important role in DNA repair and is one of the markers of mitochondria-mediated apoptosis [28]. Moreover, eriodictyol induces apoptosis through cleavage of PARP in human hepatocellular carcinoma Hep-G2 cells [23]. Our results are consistent with studies carried out previous. Eriodictyol

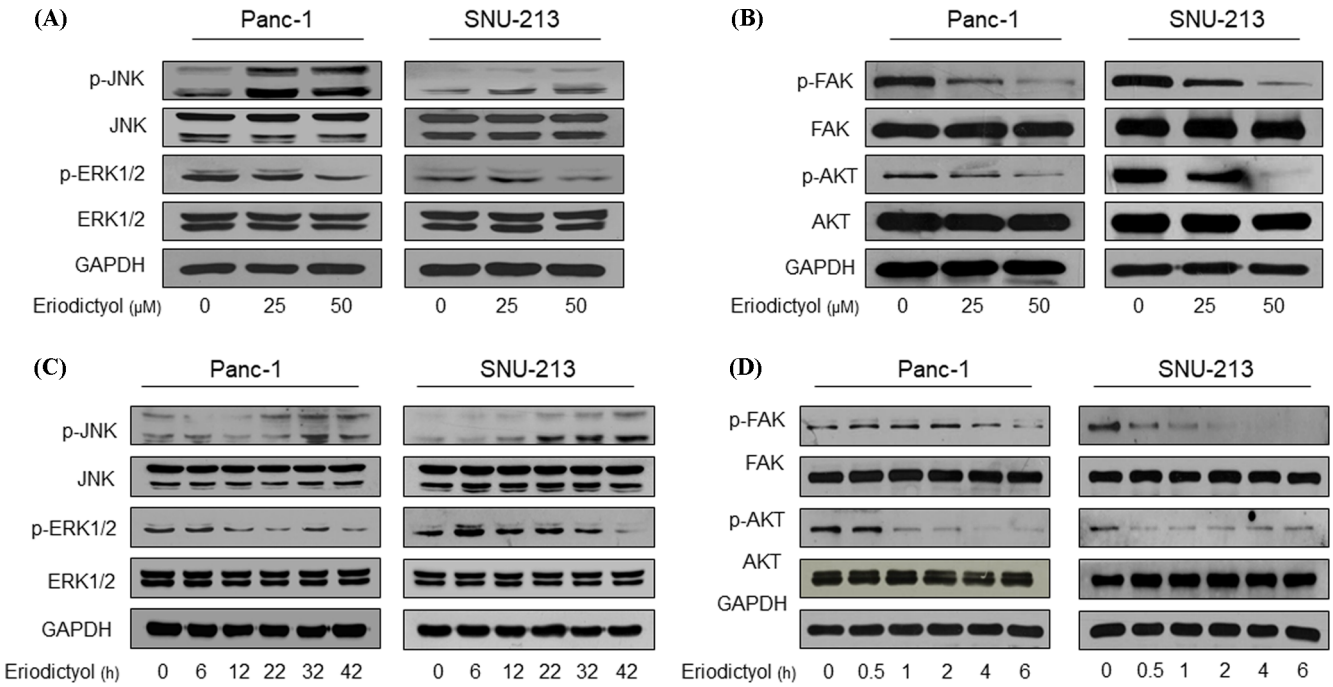

Fig. 4 Effects of eriodictyol on phosphorylation level of JNK, ERK, FAK, and AKT in pancreatic cancer cells. Western blot analysis for total or phosphorylated forms of (A) JNK, ERK1/2, (B) FAK, AKT, and GAPDH after various concentrations (0, 25, and 50 μM) of eriodictyol for 24 h in Panc-1 and SNU-213 cells. (C) Panc-1 and SNU-213 cells were treated with 50 μM eriodictyol for 0, 6, 12, 22, 32, or 42 h. Cell lysates were analyzed by SDS-PAGE, following by western blotting with antibodies against p-JNK, JNK, p-ERK1/2, ERK1/2, and GAPDH. (D) Panc-1 and SNU-213 cells were treated with 50 μM eriodictyol for 0, 0.5, 1, 2, 4, or 6 h and cells were lysed, and the indicate proteins were detected by western blotting. p-FAK, FAK, p-AKT, AKT, and GAPDH were detected by ECL. Band intensities were measured using the ImageJ software

induced cleavage of PARP in pancreatic cancer cells in a dose-dependent manner. The level of cleaved PARP and percentage of apoptotic cells increased more rapidly in SNU-213 and Panc-1 cells, indicating that PARP cleavage is closely connected to cell death in pancreatic cancer cells. Although Panc-1 cells are drug-resistant [32], eriodictyol decreased their viability and effectively inhibited anchorage-independent growth on soft agar. In addition, eriodictyol inhibited the phosphorylation of FAK/AKT, which can be assumed to promote apoptosis and inhibit cell growth, proliferation and motility [33]. Indeed, FAK activation of PI3K/AKT signaling pathway is associated with resistance to apoptosis induced by UV irradiation and TRAIL [34]. Sustained activation of JNK by treatment with several anticancer agents increases the rate of apoptosis in pancreatic cancer cells [35-37]. As with JNK activation in previous studies, eriodictyol induced sustained and elevated JNK phosphorylation, leading to apoptosis. Furthermore, the phosphorylation level of ERK, which contributes significantly to tumor formation, also decreased following eriodictyol treatment. Recent work showed that eriodictyol induces apoptosis through the PI3K/AKT signaling pathway in malignant brain tumor cells [38]. These results are consistent with previous studies that eriodictyol were found to inhibit cancer cell growth and induce apoptosis through the PI3K/AKT and JNK signaling pathway. Because eriodictyol has a relatively strong antioxidant effect and has few side effects to the human body, its anticancer effects merit further study.

In summary, this study showed that eriodictyol inhibits proliferation and induces apoptosis in pancreatic cancer cells. In additions, our findings provide insight into the molecular mechanism of eriodictyol-induced apoptosis in pancreatic cancer cells, and should thus contribute to the development of candidate reagents for the treatment of pancreatic cancer.

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